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# Osmoprotective Effect of Glycine Betaine on Thrombopoietin Production in Hyperosmotic Chinese Hamster Ovary Cell Culture: Clonal Variations

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*Accepted for publication August 14, 2000.*

**Abstract:**

When 23 recombinant Chinese hamster ovary (rCHO) cell clones were cultivated in hyperosmolar medium resulting from NaCl addition (533 mOsm/kg), their specific thrombopoietin (TPO) productivity ($q_{TPO}$) was increased. However, due to depressed cell growth at elevated osmolality, no enhancement in the maximum TPO titer was made in batch cultures of all 23 clones. To test the feasibility of using glycine betaine, known as a strong osmoprotective compound, for improved TPO production in hyperosmotic rCHO cell cultures, hyperosmotic batch cultures of 23 clones were performed in the presence of 15 mM glycine betaine. Glycine betaine was found to have a strong osmoprotective effect on all 23 clones. Inclusion of 15 mM glycine betaine in hyperosmolar medium enabled 22 clones to grow at 542 mOsm/kg, where most clones could not grow in the absence of glycine betaine, but at a cost of reduced $q_{TPO}$. However, the relative decrease in $q_{TPO}$ varied significantly among clones. Thus, efficacy of the simultaneous use of hyperosmotic pressure and glycine betaine as a means to improve foreign protein production was variable among clones. Six out of 23 clones displayed more than a 40% increase in the maximum TPO titer in the hyperosmolar medium containing glycine betaine, compared with that in the standard medium with a physiological osmolality. Taken together, the results obtained here emphasize the importance of selection of clones for the successful use of hyperosmotic pressure and glycine betaine as an economical means to improve TPO production.

## Introduction

Hyperosmotic pressure, which can be induced by addition of cheap salts to media, has been recognized as being an economical solution to increase the specific antibody productivity in hybridoma cell cultures. However, since cell growth is depressed at elevated osmolality, the use of hyperosmolar media in batch cultures did not increase the maximum antibody concentration substantially (*1-8*). To overcome this

drawback, several strategies such as adaptation of cells to hyperosmotic pressure (2, 3), use of osmoprotectants in media (5, 6), and two-stage culture (9) have been applied for improved antibody production.

Although Chinese hamster ovary (CHO) cells are popular mammalian hosts for the commercial production of therapeutically important proteins (10-12), there are, to date, few studies on the effect of hyperosmotic pressure on recombinant CHO (rCHO) cells except for a rCHO cell line (4B3) (13).

Among various strategies to overcome depressed cell growth at elevated osmolality, the use of osmoprotectants such as glycine betaine, known as a strong osmoprotective compound (5), is probably most feasible for improved foreign protein production because one can simply add glycine betaine to the culture medium.

In fact, the use of glycine betaine was found to improve cell growth of the rCHO cell line (4B3) at elevated osmolality without effect on the specific productivity ($q$) (13). Contrary to this observation, we recently observed that the use of glycine betaine improved cell growth of other rCHO cell lines (CS13-1.00* and CS13-0.02*) at elevated osmolality but at a cost of significantly reduced $q$ (14). Thus, efficacy of the simultaneous use of hyperosmotic pressure and glycine betaine as a means to improve foreign protein production appears to be variable among different rCHO cell lines.

Different responses of rCHO cell lines to hyperosmotic pressure and glycine betaine in regard to $q$ might occur because the rCHO cells resulting from transfection have different sites of integration and their subsequent consequences on host cell function may differ significantly. If so, the selection of suitable clones that retain enhanced $q$ in the presence of glycine betaine will allow the successful use of hyperosmotic pressure and glycine betaine as an economical means to improve foreign protein production.

Here, to determine the clonal variations in regard to the response of rCHO cells to hyperosmotic pressure and glycine betaine, hyperosmolar batch cultures of 23 rCHO clones producing thrombopoietin (TPO) were carried out in the absence and presence of 15 mM glycine betaine.

## Materials and Methods

**Cell Lines**. Parental CHO cells expressing a TPO were made by transfecting TPO expression plasmids into dihydrofolate reductase (DHFR)-deficient CHO cells (DUKX-B11, ATCC CRL-9096).

High-producer parental clones were subjected to successive rounds of selection in medium containing stepwise increments of methotrexate (MTX, Sigma, St. Louis, MO) levels. Nineteen amplified clones at 0.08 $\mu$M MTX used in this study were acquired as shown in Figure 1. T20-0.08 cells were plated at 0.3-0.8 cells/well in 96-well tissue-culture plates (Nunc, Roskilde) containing Iscove's modified Dulbecco's medium (IMDM) with 10% dialyzed fetal bovine serum (dFBS, Gibco, Grand Island, NY) and 0.08 $\mu$M MTX. Four high-producer subclones (T20-sub1, T20-sub2, T20-sub3 and T20-sub4) were isolated and used in this study.
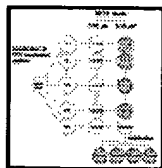


Figure 1 Schematic representation of the procedure for the establishment of TPO-producing clones. Twenty three clones used in this study are shaded.

**Culture Maintenance, Medium, and Culture**. The medium for culture maintenance of 23 clones was IMDM supplemented with 10% (v/v) dFBS and 0.08 $\mu$M MTX. No antibiotics were added to the medium. The cells were maintained as monolayer cultures in 25 $cm^2$ T flasks (Nunc) in a humidified 5% $CO_2$ incubator at 37 °C. The cells were passed every 3-4 days upon reaching confluency.

Three different media were used for batch cultures. The standard medium with a physiological osmolality was IMDM supplemented with 10% (v/v) dFBS and 0.08 $\mu$M MTX. The hyperosmolar culture medium was prepared by addition of NaCl into the standard medium. The osmoprotective medium was prepared by adding glycine betaine (Sigma) to the hyperosmolar medium at the concentration of 15 mM. The osmolalities of standard and hyperosmolar IMDM were 279 ± 7 and 533 ± 12 mOsm/kg. The addition of glycine betaine (15 mM) to the hyperosmolar medium elevated the medium osmolality by approximately 9 mOsm/kg.

Identical batch cultures using three different media were carried out with 23 clones. Cells exponentially growing in the standard medium were inoculated into 6-well tissue-culture plates containing 5 mL of media. The initial cell concentration was approximately $0.3 \times 10^5$ cells/mL. One well was sacrificed every 24-36 h for determination of cell concentration. The supernatant was aliquoted and kept frozen at -20 °C for TPO assay. The cultures were performed in a humidified 5% $CO_2$ incubator at 37 °C.

**Analytical Methods**. Cell concentration was estimated using a hemocytometer. Viable cells were distinguished from dead cells by the trypan blue dye exclusion method. Error for replicate measurements ($n = 3$) of the same sample is ± 5%.

Osmolality was measured using an osmometer (automatic semi-micro osmometer, model A0300, Knauer, Berlin). Measurement error is ± 1%.

Secreted TPO was quantified using an enzyme-linked immunosorbent assay (ELISA) according to the protocol provided by R&D Systems (Minneapolis, MN). Measurement error is ± 10%.

**Evaluation of Specific Growth and TPO Production Rates**. The specific growth rate ($\mu$) and specific TPO productivity ($q_{TPO}$) were based on data collected during the exponential growth phase and were evaluated as described earlier (*15*).

**Results**

To determine the effects of hyperosmotic pressure itself and glycine betaine addition (15 mM) to hyperosmolar medium on rCHO cells in regard to growth and TPO production, batch cultures of 23 rCHO clones selected at 0.08 $\mu$M MTX were carried out over a period of 7-8 days using three different media. Regardless of the media used, the osmolality did not change significantly during the batch culture. The concentration of glycine betaine used in this study, 15 mM, yielded the maximum osmoprotection in hyperosmotic rCHO cell cultures (*14*). When glycine betaine was added to the standard medium with a physiological osmolality, no significant effect on cell growth and TPO production was observed (data not shown). On the other hand, clonal variations in regard to osmoprotective effect of glycine betaine on TPO production in the hyperosmolar medium were found to be significant.

Figure 2 shows typical cell growth and TPO production profiles during batch cultures of clones that displayed enhanced TPO titer in the osmoprotective medium containing 15 mM glycine betaine. The

cultures were performed three separate times. In a standard medium with a physiological osmolality (279 mOsm/kg), the maximum viable cell concentration and $\mu$ of T8-0.08 clone were $1.26 \times 10^6$ cells/mL and 0.58 day$^{-1}$, respectively. In the hyperosmolar medium (533 mOsm/kg), no cell growth was observed (Figure 2A). However, addition of glycine betaine to the hyperosmolar medium improved cell growth to a great extent, though it increased the medium osmolality further by approximately 9 mOsm/kg. The $\mu$ and maximum viable cell concentration in the osmoprotective medium with 15 mM glycine betaine (542 mOsm/kg) were 86% and 34% of those in the standard medium, respectively.
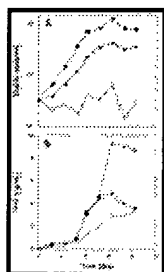


Figure 2 Batch cultures of T8-0.08 clone using three different media. (A) Viable cell concentration and (B) TPO concentration. ($\cdot$): standard medium, 279 mOsm/kg. (○): hyperosmolar medium (168 mM NaCl added), 533 mOsm/kg. (▼): osmoprotective medium (168 mM NaCl and 15mM glycine betaine added), 542 mOsm/kg.

T8-0.08 clone displayed enhanced $q_{TPO}$ in the hyperosmolar medium. The $q_{TPO}$ in the hyperosmolar medium was increased by 815%, as compared with $q_{TPO}$ in the standard medium. However, since cells did not grow in the hyperosmolar medium, the maximum TPO concentration obtained in the hyperosmolar medium was only 3.33 $\mu$g/mL (Figure 2B).

Addition of glycine betaine to the hyperosmolar medium did decrease $q_{TPO}$ significantly, but this $q_{TPO}$ (=8.88 $\mu$g/10$^6$cells/day) was still significantly higher than $q_{TPO}$ (=1.69 $\mu$g/10$^6$cells/day) in the standard medium. As a result, the maximum TPO concentration in the osmoprotective medium was 9.28 $\mu$g/mL, which was increased by 91% over that obtained in the standard medium.

Figure 3 shows typical cell growth and TPO production profiles during batch cultures of clones that did not display enhanced TPO titer in the osmoprotective medium containing 15 mM glycine betaine. The cultures were performed three separate times. Like T8-0.08 clone, cell growth of T16-0.08 clone was depressed significantly in the hyperosmolar medium (Figure 3A). Cell growth in the hyperosmolar medium could be improved significantly by addition of glycine betaine. The $\mu$ and maximum viable cell concentration in the osmoprotective medium were 75% and 48% of those in the standard medium, respectively.
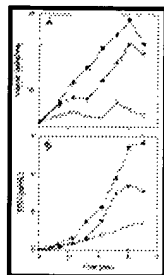


Figure 3 Batch cultures of T16-0.08 clone using three different media. (A) Viable cell concentration and (B) TPO concentration. ($\cdot$): standard medium, 279 mOsm/kg. (○): hyperosmolar medium (168 mM NaCl added), 533 mOsm/kg. (▼): osmoprotective medium (168 mM NaCl and 15mM glycine betaine added), 542 mOsm/kg.

T16-0.08 clone also displayed enhanced $q_{TPO}$ in the hyperosmolar medium. The $q_{TPO}$ in the hyperosmolar medium was increased by 87%, as compared with $q_{TPO}$ in the standard medium. However, since cells did not grow in the hyperosmolar medium, the maximum TPO concentration obtained in the hyperosmolar medium was only 1.45 $\mu$g/mL (Figure 3B). Addition of glycine betaine to the hyperosmolar medium increased the maximum TPO titer to 3.39 $\mu$g/mL. However, this titer is significantly lower than that (=5.66 $\mu$g/mL) in the standard medium. Unlike the T8-0.08 clone, osmoprotective effect of glycine betaine decreased the $q_{TPO}$ of the T16-0.08 clone to the level of $q_{TPO}$ in the standard medium. Thus, the effect of glycine betaine inclusion in the hyperosmolar medium on improved TPO production was found to be clone-specific.

Figure 4 shows the effects of hyperosmotic pressure itself and glycine betaine addition to the hyperosmolar medium on all 23 clones in regard to growth. The $\mu$ of clones in the standard medium was in the range of 0.37-0.79 day$^{-1}$. In the hyperosmolar medium, cell growth of all 23 clones was depressed significantly. Only 2 clones out of 23 clones could grow in the hyperosmolar medium.
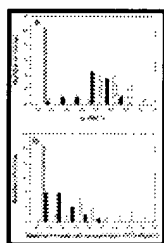


Figure 4 In three different media, $\mu$ and maximum viable cell concentration of 23 clones. (A) $\mu$ and (B) maximum viable cell concentration. Open bar: standard medium, 279 mOsm/kg. Shaded bar: hyperosmolar medium (168 mM NaCl added), 533 mOsm/kg. Closed bar: osmoprotective medium (168 mM NaCl and 15mM glycine betaine added), 542 mOsm/kg. The numbers of clones in parts A and B correspond to a range of $\mu$ and maximum viable cell concentration, respectively.

Glycine betaine was found to have strong osmoprotective effects. Addition of glycine betaine to the hyperosmolar medium improved cell growth of 22 clones, though the degree of improvement varied among clones. However, regardless of clones, $\mu$ and maximum viable cell concentration obtained in the osmoprotective medium were still lower than those obtained in the standard medium. The $\mu$ and maximum viable cell concentration of 23 clones in three different media were summarized in Table 1.🔲 Figure 5 shows the effects of hyperosmotic pressure itself and glycine betaine addition to the hyperosmolar medium on all 23 clones in regard to TPO production. The $q_{TPO}$ of clones in the standard medium was in the range of 0.30-8.37 $\mu$g/10$^6$cells/day. In the hyperosmolar medium, all 23 clones displayed enhanced $q_{TPO}$ and their $q_{TPO}$ were in the range of 1.33-28.55 $\mu$g/10$^6$ cells/day (Figure 5A). However, their maximum TPO titer obtained in the hyperosmolar medium was significantly lower than that in the standard medium because of depressed cell growth at elevated osmolality (Figure 5B).
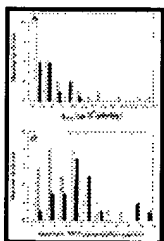


Figure 5 In three different media, $q_{TPO}$ and maximum TPO concentration of 23 clones. (A) $q_{TPO}$ and (B) maximum TPO concentration. Open bar: standard medium, 279 mOsm/kg. shaded bar: hyperosmolar medium (168 mM NaCl added), 533 mOsm/kg. Closed bar: osmoprotective medium (168 mM NaCl and 15mM glycine betaine added), 542 mOsm/kg. The numbers of clones in parts A and B correspond to a range of $q_{TPO}$ and maximum TPO concentration, respectively.

Contrary to the common osmoprotective effects of glycine betaine on cell growth, the effects on TPO production due to the addition of glycine betaine to the hyperosmolar medium differed significantly among clones (Figure 5B). For six clones, the maximum TPO titer in the osmoprotective medium was increased by more than 40%, compared with that in the standard medium. Thus, the highest TPO titer could be obtained in the osmoprotective medium (Figure 5B). On the other hand, the maximum TPO titer of some clones in the osmoprotective medium was even lower than that in the standard medium, because their $q_{TPO}$ in the osmoprotective medium was similar to that in the standard medium. The $q_{TPO}$ and maximum TPO concentration of 23 clones in three different media were also summarized in Table 1.

**Discussion**

Among various strategies, a simultaneous use of hyperosmotic pressure and osmoprotective compound is thought to be one of the most feasible means for improved foreign protein production in rCHO cell cultures because of easy implementation and low cost. The osmoprotective medium is prepared simply by adding NaCl (168 mM) and glycine betaine (15 mM) to the medium. This addition increases the price of medium by approximately 22 cents/L of medium (Calculation was made based on the price listed in the 2000 Sigma catalog).

As is the case with the hybridoma cell line 6H11 (5, 6), inclusion of NaCl and glycine betaine in the culture medium may increase foreign protein product titer in rCHO cell cultures. If so, it will result in a great impact on economical foreign protein production from rCHO cell cultures. Thus, in an effort to improve TPO production in rCHO cells, effects of hyperosmotic pressure itself and glycine betaine addition to the hyperosmolar medium on rCHO cells in regard to TPO production were determined in this study. Since the rCHO cells resulting from random integration of a foreign gene into their chromosomes have different sites of integration and their subsequent consequence on host cell function may differ significantly, 23 clones were used in this study.

All 23 clones displayed increased $q_{TPO}$ in the hyperosmolar medium. However, due to a simultaneous depression of cell growth, no improvement in the maximum TPO titer was caused by use of hyperosmotic pressure alone.

Glycine betaine was found to have a strong osmoprotective effect on 22 clones under hyperosmotic pressure. Inclusion of 15 mM glycine betaine in hyperosmolar medium enabled 22 clones to grow at 542 mOsm/kg, where most clones could not grow in the absence of glycine betaine.

Glycine betaine is likely to be transported into cells under hyperosmotic pressure (16-18). Glycine betaine accumulated inside the cells may balance the increased extracellular osmolality so that the cells can avoid unfavorable pressure differences across the membrane (18). Further, it may also create an environment where the structure of cellular macromolecules is stabilized thermodynamically (19, 20). This environment may allow cells to resume proliferation. However, the detailed mechanism of the osmoprotective effect of glycine betaine needs to be elucidated clearly at the basic cellular level.

Contrary to the common osmoprotective effects of glycine betaine on cell growth, addition of glycine betaine to the hyperosmolar medium exerted different influences on TPO production. Addition of glycine betaine to the hyperosmolar medium decreased $q_{TPO}$ of all 23 clones at a cost of cell growth, but the degree of decrease in $q_{TPO}$ varied significantly among 23 clones. For some clones, the maximum TPO titer in the osmoprotective medium was even lower than that in the standard medium. However, 6 out of 23 clones displayed more than a 40% increase in the maximum TPO titer in the osmoprotective medium, compared with that in the standard medium. Furthermore, a TPO titer higher than 8 μg/mL could be

obtained only in the osmoprotective medium (Figure 5B).

The response of clones to glycine betaine addition in regard to $q_{TPO}$ may depend on the integration sites of TPO gene. The observation that four subclones (T20-sub1, T20-sub2, T20-sub3, and T20-sub4) derived from the same parental clone displayed a response similar to that of glycine betaine addition in regard to $q_{TPO}$ supports this hypothesis. Similarly, it has been suggested that the effect of butyrate treatment on expression of specific target genes in CHO and monkey kidney cells may be integration-site-dependent (21, 22). However, the detailed mechanism of the effect of glycine betaine on $q_{TPO}$ at the molecular level remains to be elucidated.

The clonal variations in response to glycine betaine addition in regard to TPO production are indicative of the importance of clone selection for manufacturing. In many cases, a cell line is established based on clones with high productivity, and cell culture process development for manufacturing is carried out using that cell line. However, if a specific cell culture process is in mind, clones suitable for that process should be selected because of clonal variability.

In conclusion, addition of glycine betaine to the hyperosmolar medium exerted different influences on TPO production. If the proper clones are selected, the use of osmoprotective medium containing NaCl and glycine betaine in batch culture increases the maximum TPO titer significantly, compared with the standard medium. Furthermore, the use of glycine betaine may also be effective for improved TPO production in fed-batch culture. Culture longevity in fed-batch culture is often limited by elevated medium osmolality caused by repeated nutrient feeding. The use of glycine betain is likely to improve the culture longevity in fed-batch culture. Taken together, the prerequisite of a successful use of NaCl and glycine betaine for improved TPO production in rCHO cell cultures is the selection of suitable clones that can retain enhanced $q_{TPO}$ in the presence of glycine betaine.

## Acknowledgment

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Table 1. Effect of Hyperosmotic Pressure and Glycine Betaine Addition on $\mu$, $q_{TPO}$, Maximum Viable Cell Concentration and Maximum TPO Concentration in Batch Culture of 23 Clones Using Three Different Media

| clone | media[a] | $\mu$ (day$^{-1}$) | $q_{TPO}$ ($\mu$g/10$^6$ cells/day) | max viable cell concn (10$^6$ cells/mL) | max TPO concn ($\mu$g/mL) |
|---|---|---|---|---|---|
|  | standard | 0.55 | 0.70 | 1.67 | 2.58 |
| T1-0.08 | hyperosmolar | 0.38 | 2.98 | 0.33 | 2.45 |
|  | osmoprotective | 0.45 | 2.12 | 0.68 | 3.42 |
|  | standard | 0.61 | 1.64 | 1.42 | 4.27 |
| T2-0.08 | hyperosmolar | NG[b] | 4.80 | 0.03 | 2.52 |
|  | osmoprotective | 0.44 | 2.92 | 0.61 | 4.93 |
|  | standard | 0.42 | 2.19 | 0.69 | 3.39 |
| T3-0.08 | hyperosmolar | NG | 10.33 | 0.03 | 2.68 |
|  | osmoprotective | 0.25 | 6.82 | 0.19 | 5.41 |
|  | standard | 0.79 | 8.37 | 0.59 | 7.53 |
| T4-0.08 | hyperosmolar | NG | 21.76 | 0.03 | 3.86 |
|  | osmoprotective | 0.41 | 11.86 | 0.22 | 8.41 |
|  | standard | 0.51 | 4.33 | 0.34 | 3.54 |
| T5-0.08 | hyperosmolar | NG | 28.55 | 0.03 | 1.62 |
|  | osmoprotective | 0.31 | 7.68 | 0.10 | 3.47 |
|  | standard | 0.51 | 4.07 | 0.65 | 6.00 |
| T6-0.08 | hyperosmolar | NG | 5.20 | 0.03 | 3.48 |
|  | osmoprotective | 0.41 | 4.12 | 0.34 | 4.94 |
|  | standard | 0.48 | 0.58 | 1.40 | 3.10 |
| T7-0.08 | hyperosmolar | 0.24 | 3.14 | 0.23 | 3.47 |
|  | osmoprotective | 0.37 | 2.75 | 0.48 | 4.08 |
|  | standard | 0.54 ± 0.07 | 1.59 ± 0.17 | 0.85 ± 0.49 | 3.26 ± 1.87 |
| T8-0.08[c] | hyperosmolar | NG | 15.67 ± 2.77 | 0.01± 0.02 | 1.27 ± 1.79 |
|  | osmoprotective | 0.44 ± 0.18 | 10.77 ± 1.90 | 0.21 ± 0.20 | 5.13 ± 3.91 |
|  | standard | 0.44 | 0.40 | 1.53 | 3.17 |
| T9-0.08 | hyperosmolar | NG | 15.60 | 0.03 | 0.46 |
|  | osmoprotective | 0.41 | 1.54 | 0.93 | 8.29 |
|  | standard | 0.60 | 0.30 | 1.27 | 2.57 |

| T10-0.08 | hyperosmolar | NG | 27.20 | 0.03 | 1.65 |
|---|---|---|---|---|---|
| | osmoprotective | 0.45 | 1.45 | 0.47 | 4.24 |
| | standard | 0.37 | 3.09 | 0.26 | 2.32 |
| T11-0.08 | hyperosmolar | NG | 10.59 | 0.03 | 1.95 |
| | osmoprotective | 0.34 | 7.08 | 0.16 | 3.34 |
| | standard | 0.53 | 1.21 | 1.00 | 3.88 |
| T12-0.08 | hyperosmolar | NG | 1.55 | 0.03 | 0.63 |
| | osmoprotective | 0.23 | 1.49 | 0.23 | 1.74 |
| | standard | 0.53 | 2.59 | 0.93 | 5.68 |
| T13-0.08 | hyperosmolar | NG | 4.53 | 0.03 | 1.42 |
| | osmoprotective | 0.37 | 3.12 | 0.37 | 3.34 |
| | standard | 0.61 | 1.99 | 0.91 | 3.24 |
| T14-0.08 | hyperosmolar | NG | 2.35 | 0.03 | 0.67 |
| | osmoprotective | 0.34 | 3.84 | 0.20 | 3.20 |
| | standard | 0.46 | 2.97 | 0.61 | 4.07 |
| T15-0.08 | hyperosmolar | NG | 5.98 | 0.03 | 2.03 |
| | osmoprotective | 0.44 | 2.35 | 0.22 | 2.52 |
| | standard | $0.56 \pm 0.04$ | $2.84 \pm 0.51$ | $0.75 \pm 0.12$ | $4.36 \pm 1.47$ |
| T16-0.08[c] | hyperosmolar | NG | $7.89 \pm 2.94$ | $0.01 \pm 0.01$ | $0.61 \pm 0.73$ |
| | osmoprotective | $0.35 \pm 0.08$ | $2.62 \pm 0.32$ | $0.20 \pm 0.19$ | $2.05 \pm 1.16$ |
| | standard | 0.53 | 5.90 | 0.34 | 3.88 |
| T17-0.08 | hyperosmolar | NG | 12.70 | 0.03 | 2.19 |
| | osmoprotective | 0.33 | 9.19 | 0.11 | 4.05 |
| | standard | 0.48 | 2.06 | 0.83 | 3.67 |
| T18-0.08 | hyperosmolar | NG | 7.01 | 0.03 | 1.70 |
| | osmoprotective | 0.30 | 4.47 | 0.15 | 3.43 |
| | standard | 0.48 | 0.39 | 0.66 | 0.76 |
| T19-0.08 | hyperosmolar | NG | 1.33 | 0.03 | 0.43 |
| | osmoprotective | 0.17 | 1.95 | 0.13 | 1.08 |
| | standard | 0.46 | 1.56 | 0.70 | 2.77 |
| T20-sub1 | hyperosmolar | NG | 3.39 | 0.03 | 1.29 |
| | osmoprotective | 0.32 | 3.12 | 0.23 | 2.02 |
| | standard | 0.64 | 0.85 | 0.71 | 2.01 |
| T20-sub2 | hyperosmolar | NG | 5.44 | 0.03 | 1.58 |
| | osmoprotective | 0.54 | 1.99 | 0.25 | 2.35 |
| | standard | 0.48 | 0.65 | 0.57 | 0.96 |
| T20-sub3 | hyperosmolar | NG | 2.44 | 0.03 | 0.75 |

|  |  |  |  |  |  |
|---|---|---|---|---|---|
|  | osmoprotective | 0.11 | 1.77 | 0.15 | 1.16 |
|  | standard | 0.60 | 2.39 | 0.12 | 1.41 |
| T20-sub4 | hyperosmolar | NG | 4.68 | 0.03 | 0.18 |
|  | osmoprotective | NG | 9.63 | 0.02 | 0.37 |

[a] The osmolalities of standard, hyperosmolar and osmoprotective media were 279, 533, and 542 mOsm/kg, respectively. The osmoprotective medium was prepared by adding glycine betaine to the hyperosmolar medium at a concentration of 15 mM. [b] NG stands for no growth. [c] Means± standard deviation. This experiment was performed three separate times.